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AWARD NUMBER: DAMD17-03-1-0402

TITLE: Selective Inhibition of T Cell Tolerance as a Means of Enhancing Tumor Vaccines in a Mouse Model of Breast Cancer

PRINCIPAL INVESTIGATOR: Jonathan Powell, M.D., Ph.D.

CONTRACTING ORGANIZATION: Johns Hopkins University School of Medicine
Baltimore, Maryland 21205

REPORT DATE: June 2006

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE				<i>Form Approved</i> OMB No. 0704-0188	
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1. REPORT DATE (DD-MM-YYYY) 01-06-2006		2. REPORT TYPE Final		3. DATES COVERED (From - To) 15 May 2003 – 14 May 2006	
4. TITLE AND SUBTITLE Selective Inhibition of T Cell Tolerance as a Means of Enhancing Tumor Vaccines in a Mouse Model of Breast Cancer				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER DAMD17-03-1-0402	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Jonathan Powell, M.D., Ph.D. E-Mail: poweljo@jhmi.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Johns Hopkins University School of Medicine Baltimore, Maryland 21205				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Purpose: To determine if the addition of Go6976 to vaccine protocols will inhibit neu specific tolerance and enhance immunotherapy for breast cancer. Scope: In the Her-2/neu model of spontaneous breast cancer the immune system of these transgenic mice are tolerant to the neu protein. While immunity to neu can be demonstrated in the neu-transgenic mice (partial breaking of tolerance), this immunity is inadequate to prevent the spontaneous development of tumors and to prevent death from tumor challenge. Findings: By combining our regimen with a dose of cytoxan we can promote survival of tumor bearing mice when compared with no treatment, vaccine alone or vaccine + cytoxan. In particular, this combination is very effective in inhibiting tumor growth in the early period post-tumor challenge. Unfortunately, during the last year efforts to improve long term survival have not been successful. Significance: These data support the notion that the novel combination of PKC inhibitor + vaccine can enhance the efficacy of tumor vaccines. More work needs to be done to optimized the dosing schedule of this approach.					
15. SUBJECT TERMS Breast cancer, vaccine, immunology					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 8	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

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Introduction:

In the Her-2/*neu* model of spontaneous breast cancer development it is clear that the immune system of these transgenic mice are tolerant to the neu protein (1-3). In this model not only does the overexpression of neu lead to tumorigenesis but the neu protein is the target of both humoral and cellular immunity which prevent tumor-induced death in the non-transgenic mice (1, 4,5). Indeed, while immunity to neu can be demonstrated in the neu-transgenic mice (partial breaking of tolerance), this immunity is inadequate in terms of preventing the spontaneous development of tumors and preventing death from tumor challenge. We have demonstrated *in vitro* that the PKC inhibitor Go6976 has the ability to selectively inhibit TCR induced tolerance induction while only minimally inhibiting T cell activation. We hypothesize that the addition of Go6976 to vaccine protocols will inhibit the reinduction of neu specific tolerance and thus facilitate immune mediated protection against the development of spontaneous breast cancer development and tumor challenge.

Body:

Initial studies were preformed in order to determine the optimal dosing and kinetics of the administration of Go6976 in relation to the tumor vaccine. In as much as this is a novel approach to tumor immunotherapy there was no precedent to guide us. Based on studies employing PKC inhibitors *in vivo* for other purposes we started with a dose of 5mg/kg q3 days for 3 doses. The mice were treated as follows:

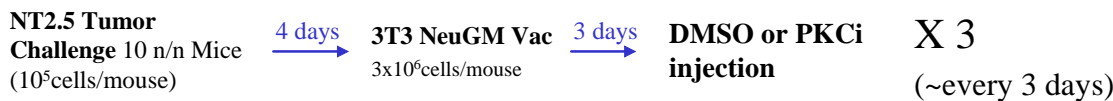


Figure 1

We treated 10 mice in the DMSO group and 10 in the Go6976 (PKCi) group. At approximately day 30 both sets of mice had developed tumors with the exception of one mouse in the treated group. At this time, lymphocytes were harvested from the spleens of both sets of mice. The cells were restimulated *in vitro* and then assessed for activation by intracellular staining for IFN- γ . Only the mouse from the treated group displayed evidence of a neu-specific T cell response (Figure 2). Of note, none of the Go6976 treated mice displayed any adverse effects of the drug. While these data are not dramatic, we interpreted them as an indication that the drug might have had a positive immunologic effect. More importantly, it provided a framework on which to build a more effective dosing regimen.

Based upon these initial experiments we doubled the dose of the drug to 10mg/Kg IP q3 days this time for 4 doses. As seen in Figure 3 beginning at about day 18 we saw a difference in the development of tumor between the untreated and Go6976 treated groups. By day 24 this difference was less pronounced. Note, even at this higher dose there was no evidence of toxicity. Once again we interpreted these data as indicating that the Go6976 was potentially delaying the induction of neu specific tolerance. The fact that the effect was lost indicated that we might need to maintain the PKCi treatment longer and perhaps decrease the dosing interval.

Figure 3: The mice were treated as described in Figure 1 with the exception that the dose was increased to 10mg/Kg IP q3 days for 4 doses.

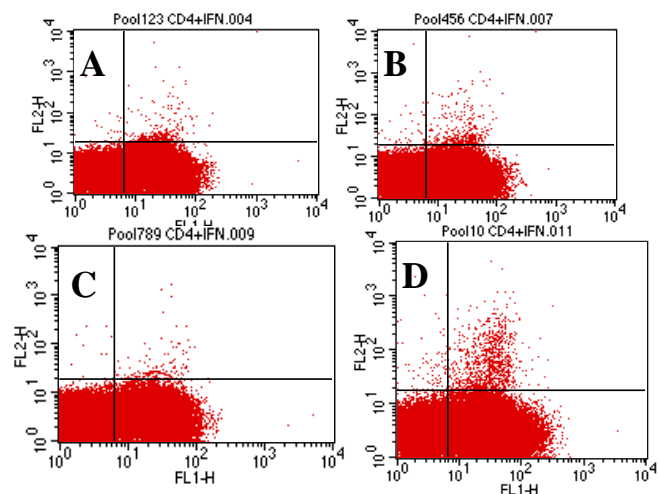
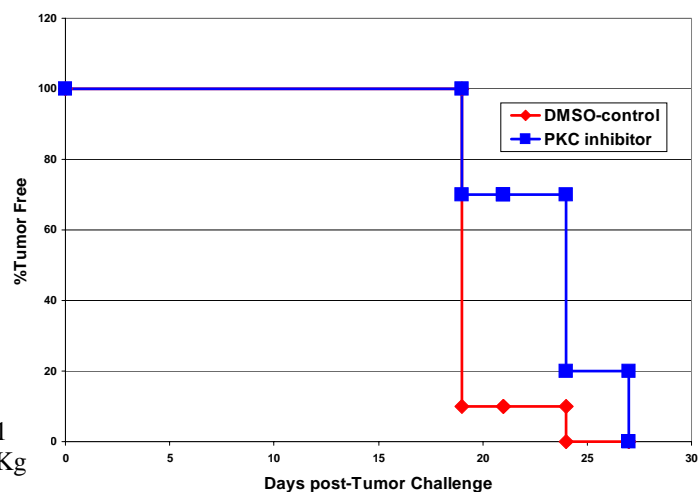
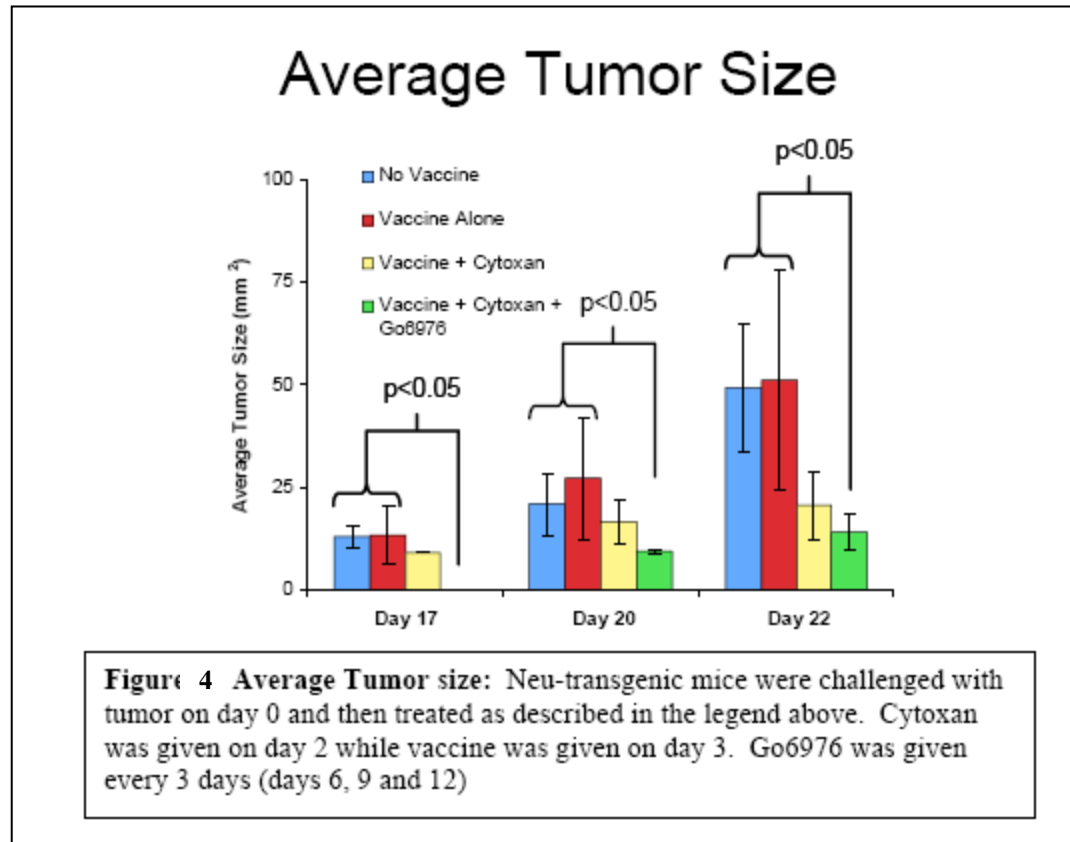


Figure 2: Spleens were harvested and incubated for one week with irradiated FVB/n splenocytes as APCs, and irradiated 3T3-neu cells as a source of antigen. The T cells were isolated and stimulated overnight with irradiated, IFN- γ treated 3T3-neu cells and then they were evaluated for intracellular IFN- γ production. The plots above are pooled T cells from the Go6976 treated mice in which tumors grew (A-C) and the treated mouse which rejected its tumor (D)



It is becoming increasingly clear that T regulatory cells have the ability to promote tumor-induced tolerance and inhibit vaccine function (6). This has also been found to be true in the *Neu* breast cancer model (Dr. Elizabeth Jaffee personal communication). It has long been known that cytoxan can enhance immune function and it is thought that this is due to the ability of this drug to eliminate T regulatory cells (7). Furthermore, vaccine in the setting of cytoxan has been shown to be marginally effective in this model. Thus, a series of experiments was performed to determine if Go6976 could enhance vaccine therapy + cytoxan. Mice were treated as described in Figure 1 (10 per group) with either no vaccine, vaccine alone, vaccine+cytoxan (10mg/kg IP on day 2), or vaccine+cytoxan+Go6976. The mice were evaluated for tumor size and survival.



As seen above at day 17 no tumor is evident in the Go6976 treated mice. Of note, there is a statistically significant difference in tumor growth between the vaccine+cytoxan+Go6976 group and the vaccine+cytoxan group. Thus, the effect is not due to the cytoxan but due to the addition of Go6976. The difference is still prevalent on Day 20 but loses significance on Day 22. At this time however, although tumor size in both the vaccine+cytoxan and the vaccine+cytoxan+Go6976 group are equivalent, they are still statistically smaller than the tumors in the vaccine alone group. Thus, as time goes on we lose the "Go6976 effect". Recall, the last dose of Go6976 was on day 12. Thus we interpret these findings as indicating that the addition of Go6976 can enhance vaccine efficacy (Day 17) early and that by continuing Go6976 treatment we might be able to prolong this effect.

In addition to tumor size we also examined the effect of Go6976 on survival. As seen below in Figure 5, the mice treated with vaccine+cytoxan+Go6976 had their survival curve shifted to the right when compared with the other treatments. Furthermore, the curve plateaued at 20% compared with 0% for the other groups. As mentioned above, normally the vaccine treated mice display around 15-30% survival so that for this experiment the conditions were more rigorous. Once again, the marked delay in death leads us to hypothesize that by continuing the treatment we might improve long term survival to a greater extent. We do not believe that the effect of Go6976 is due to the drug itself acting on the tumor because in previous experiments, drug alone did not have any beneficial effect (data not shown).

Tumor Free survival

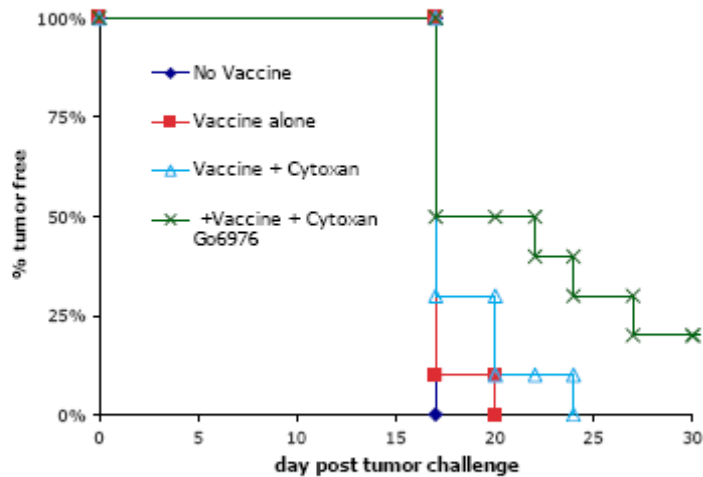


Figure 5 Tumor free survival: Mice were treated as described for Figure 2, as seen in the figure only the curve for the vaccine+cytoxin+Go6976 treated mice plateaued (20%).

The above findings were encouraging that the combination of G0 + cytoxin could immunomodulate the anti-tumor response and enhance vaccine efficacy. We reasoned that giving Go for an extended period of time might enhance its efficacy and increase tumor free survival. Specifically, the dosing of G0 was continued until day 24. Thus a series of experiments were initiated using the same model but increasing the duration of Go treatment. Figure 6 demonstrates that increasing the duration of Go treatment led to a decrease in tumor size. Interestingly, for the first time we show that Go could act independent of the vaccine. This could mean that either the Go has intrinsic anti-tumor effects or that the Go was enhancing the endogenous immune response independent of the vaccine. Unfortunately, as seen in Figure 7 this new protocol was not able to enhance overall disease free survival.

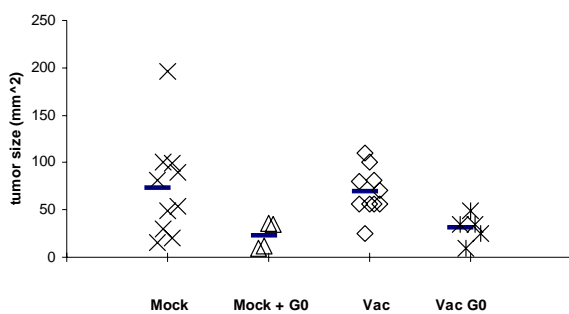


Figure 6: The mice were treated as described in the text and tumor size was determined on day 30. No vaccine (mock), No vaccine + Go6976 (Mock+GO), Vaccine (vac) and Vaccine + Go6976 (vac+GO). Note, all of the mice received cytoxin.

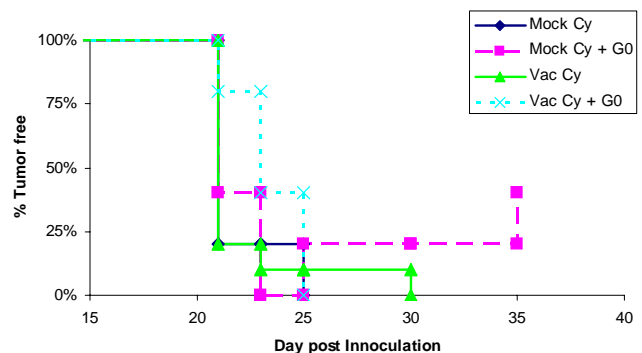


Figure 7: The mice were treated as described in the text. Note, all of the mice received cytoxin (Cy) either alone (mock, with Go6976 (+GO), with vaccine (vac) or with vaccine and Go6976.

Key Research Accomplishments:

- * Proof of the principle that the addition of Go6976 can enhance immune responses.
- * Observation that a dose of cytoxin + G06976 can synergize in terms of enhancing vaccine therapy suggesting that the combination of these two agents represent a rationale non-overlapping regimen

* Establishment of the principle of pharmacologic Tolerance Suppression Therapy as adjuvants to immunotherapy for cancer.

Reportable Outcomes:

1. Presentation of findings as a poster at "The Era of Hope" meeting 2005
2. The experiments described herein contributed in part to the PhD thesis of Paul Zarek, a student in Pharmacology and Molecular Sciences at Johns Hopkins (Note, Paul himself was not supported by the DOD but by a training grant)
3. The models established by this grant and the data supporting "Tolerance Suppression Therapy" enabled us to apply to the Flight Attendant Medical Research Institute for a grant entitled. "A2a receptor antagonism as a novel means to enhance vaccine therapy for the treatment and prevention of breast cancer".

Conclusion:

The "idea" behind this Idea Award was that immunotherapy for cancer could be enhanced by the concomitant administration of pharmacologic agents that inhibit tumor-induced T cell tolerance. In this proposal we sought to use the PKC inhibitor Go6976 to enhance immunotherapy in mice that are engineered to spontaneously develop breast cancer and thus are naturally tolerant to tumor antigens. While clearly we were able to demonstrate efficacy of G0 in enhancing immunotherapy we were not able to induce significant prolonged disease free survival. First, because in this model the mice develop spontaneous tumors it is much more representative of what happens in humans and thus sets the bar high for establishing effective treatment. Second, we were greatly hampered by our ability to perform Pharmacokinetics and thus truly optimize drug delivery. It may be that after optimizing Go levels we would increase efficacy.

The role of T regulatory cells in promoting tumor-induced tolerance is becoming greater appreciated (6). In this regard the combination of cytoxan and Go6976 appeared to be synergistic. Presumably, the cytoxan helped to eliminate T regulatory cells prior to the vaccine and then Go6976 helped to prevent the reinduction of tolerance after the vaccine. Along these lines, for the future, experiments are planned to demonstrate that the decreased tumor size and enhanced survival is due to an increase in tumor-specific T cells. In addition, we will also perform experiments to demonstrate that there is also a decrease in T regulatory cells.

Most importantly, the data derived from these studies support the concept of "Tolerance Suppression Therapy". Indeed, our lab has used the experience gained from working with Go6976 to examine other pharmacologic agents with the potential to enhance tumor vaccines. Specifically, we have been focusing on A2aR antagonists as a means of enhancing tumor vaccines. Based on our data demonstrating that A2a receptor engagement can inhibit T cell function and promote T cell tolerance AND the marked enhanced efficacy of the tumor vaccine in the A2a KO mice we propose to develop A2a specific antagonists as a means of enhancing tumor vaccine therapy. Indeed we hope that shortly we will be able to introduce such antagonists into clinical trial.

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